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Studies of rabbit testes infected with *Treponema pallidum*

I Immunopathology

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SUMMARY Rabbit testes were injected with suspensions of *Treponema pallidum,* washed *T pallidum,* heat killed *T pallidum,* or Reiter treponemes. The testes were removed three to 24 days after injection and examined for the number of treponemes, the presence of treponemal antibodies, histopathological changes, and presence of T and B cells. In animals infected with *T pallidum* a substantial number (10^6-10^7/ml) of organisms were still present at day 24 in spite of early local production of antibodies and increasing infiltration with plasma cells, T lymphocytes, and macrophages.

In animals infected with washed *T pallidum* a lower degree of inflammation was observed than in those infected with unmodified *T pallidum,* and the treponemal antibodies were detected simultaneously in samples of testicular fluid and serum. In the groups injected with heat killed *T pallidum* and Reiter treponemes no macroscopical or microscopical changes were detected, although in the group injected with heat killed *T pallidum* treponemal antibodies were detected in the testicular fluid on day 24.

Introduction

In 1978 we initiated a series of experiments on the immunopathological changes occurring in rabbit testes from days 3 to 24 after infection.[1] In those studies the most important findings were (a) the presence of B cells among the infiltrating mononuclear cells, (b) the early appearance of antitreponemal antibodies in the testes before any could be detected in sera, and (c) the persistence of treponemes long after orchitis had subsided (day 24 after infection).

In recent studies over a similar study period Lukehart *et al* observed predominantly T cells in the infiltrated testes.[2] Their finding of no treponemes at two weeks after infection and few or no B cells during infiltration prompted them to correlate the clearance of *T pallidum* from the testes with the appearance and increase in T lymphocytes.

The discrepancies between their findings and our previous report, and the importance of interpreting these differences for the understanding of relations between hosts and parasites, prompted us to re-examine the kinetics of the pathological changes occurring in the infected testes during the first four weeks of infection. We added controls not included in any of the earlier studies. In this report we concentrated on the histopathological changes, especially with regard to infiltrating T and B cells which were identified by specific labelled antibodies and mitogenic stimulation and correlated with the number of *T pallidum* in the testes during early infection. In the succeeding papers in this series we present data on local antibody production and on the inhibitory properties of the cells infiltrating the testes.[3 4]

Materials and methods

RABBITS

Adult male Nys (Flemish Giant) rabbits were obtained from the Laboratories for Veterinary Sciences of this centre. All were homozygous at the

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light chain allotypic locus (Ab4/Ab4). The animals were caged individually in air conditioned quarters (18-22°C) and given water and rabbit chow free of antibiotics (Charles River, Division of Agway, Syracuse, New York, United States).

MICRO-ORGANISMS

T pallidum (Nichols strain) was obtained from infected rabbit testes on day 9 after infection, before the peak of orchitis. Animals were killed by injection with a lethal dose of euthanasia agent T-61 (American Hoechst Corporation, Animal Health Division, Somerville, New Jersey, United States). The testes were removed aseptically and cut transversely with a scalpel. The organisms were extracted for 20 minutes at 35°C into 15 ml of sterile phosphate buffered saline (PBS) per testis. Cells and gross debris were removed by centrifugation at 200 × *g* for 10 minutes. The organisms were counted in a dark field microscope,[5] and the concentration was adjusted to the experimental needs.

The *T pallidum* suspensions from several testes were pooled and used as unmodified *T pallidum* suspension (A) containing 22 × 10^6 motile organisms/ml; suspension of washed *T pallidum* (B) obtained by washing aliquots of suspension (A) three times in excess of PBS; and suspension of heat killed *T pallidum* (C) obtained by heating aliquots of suspension (A) at 60°C for 60 minutes. The number of treponemes in suspension (C) was readjusted to 130 × 10^6 organisms/ml to compensate for the number of non-motile organisms.

Treponema phagedenis biotype Reiter (Reiter treponeme) was cultured at 37°C in spirochetal medium enriched with inactivated normal rabbit serum.[6] A three or four day old culture was washed three times with an excess of PBS, and the suspension was adjusted to 130 × 10^6 organisms/ml.

INJECTION

In the first experiment 43 rabbits were injected intratesticularly (1 ml/testis) with the following suspensions: 15 with *T pallidum;* eight with washed *T pallidum;* eight with heat killed *T pallidum;* eight with Reiter treponemes; and four with a cell free normal rabbit testis extract prepared as for the *T pallidum* suspension.

In the second experiment, to identify T and B cells in testicular infiltrate, another two groups of animals infected with *T pallidum* and normal control rabbits were used.

EXAMINATION OF TESTES

The animals were divided into four groups, each containing three or four rabbits injected with *T pallidum,* two with washed *T pallidum,* two with heat killed *T pallidum,* and one with normal rabbit testis extract. The animals were bled and killed (agent T-61) on days 3, 6, 11, or 24. The testes were removed aseptically and kept in sterile Petri dishes on ice until all rabbits of a group had been killed (about 60-80 minutes). All testes were used for counting the micro-organisms, histological examination, and measurement of treponemal antibodies.

COUNTING NUMBERS OF *T PALLIDUM*

Each testis was cut in half transversely, and duplicate imprints of each half were made on microscope slides; 5 μl PBS was placed in the centre of each imprint and covered with a coverslip. The number of treponemes was determined by examination of 50 microscope fields of each slide (eight slides per rabbit) and expressed as a mean per group of animals.[5]

HISTOPATHOLOGICAL EXAMINATION

Tissue sections 3-4 μm thick were prepared from testes fixed with formalin, stained with haematoxylin and eosin, and examined for pathological changes.

IDENTIFICATION OF TREPONEMAL ANTIBODIES IN TESTICULAR FLUID AND SERUM

Two half testes from each animal were centrifuged at 10 000 × *g* for one hour, and the testicular fluid was removed. Serum was obtained from blood taken before the rabbits were killed. The samples of testicular fluid and serum were examined by the fluorescent treponemal antibody (FTA) test in which only the first dilution of serum or fluid was prepared in sorbent (Beckman Instruments, Fullerton, California, United States) and the remaining dilutions were prepared in PBS. Slides were also commercial products (Beckman). Pretitrated goat anti rabbit IgG (reacting also with light chains) labelled with fluorescein-isothiocyanate (FITC) was used as conjugate (fluorescein:protein ratio 4:2). The incubation period of the samples of serum or fluid with conjugate was 30 minutes with intermittent washings (20 minutes) of slides in PBS.

IDENTIFICATION OF T AND B CELLS IN INFECTED TESTES

Immunofluorescence test

To identify T and B cells, individual testes were removed from eight rabbits infected with *T pallidum* at the peak of orchitis (days 9 to 12) and from two normal rabbits. The testes were immediately cut in half transversely and smeared on slides which were air dried, fixed in absolute ethanol for 60 minutes and in 95% and 75% alcohol (10 minutes each), and rinsed in PBS. Two antisera labelled with FITC were used: goat IgG $F(ab')_2$ fragment anti rabbit

immunoglobulins and bovine IgG anti rabbit thymocyte (Cappel Laboratories, Cochranville, Pennsylvania, United States). Before use both antisera were absorbed with mouse acetone liver powder prepared in our laboratory; the anti thymocyte serum was also absorbed twice with washed packed cells from normal rabbit testes. The fixed slides were flooded with conjugated antiserum for 30 minutes, washed three times with PBS, and examined in a Nikon-Optiphot microscope with an epifluorometer and a Hoffman modulation contrast system. The light source was a 75 W Xenon illuminator with an excitation filter (460-485 nm) and a barrier filter (515 nm) (Nikon Inc, Instrument Division, Garden City, New York).

Preparation of cells for mitogenic stimulation

Eight animals infected with *T pallidum* were used on days 7 to 11 after infection. The preparatory steps included extraction of leucocytes and testicular cells from the testes and selective depletion of testicular cells or leucocytes.

To extract cells, the testes were cut longitudinally several times with a scalpel and the cells extracted into RPMI (Roswell Park Memorial Institute) 1640 medium (20 ml per pair of testes) for 20 minutes. The cell suspension was centrifuged at 200 × *g* for 10 minutes, the sediment was resuspended in 30 ml of fresh medium, and the gross debris was allowed to settle for about 10 minutes. The supernatant, a suspension of primarily single cells, was transferred to another tube and centrifuged again at 200 × *g* for 10 minutes. The pelleted cells were washed twice in medium and resuspended in RPMI 1640 medium containing antibiotics and 10% inactivated fetal calf serum. The final suspension contained mononuclear leucocytes (65-80%), testicular cells, and a few spermatozoa. This mixture was called the testicular cell suspension (TCS).

To prepare TCS selectively depleted of testicular cells (TCd-TCS) or leucocytes (LCd-TCS), complement mediated cytotoxicity tests were performed with antisera raised in goats against rabbit thymus cells and against rabbit testicular cells. Goat anti rabbit thymus serum (AThS) was prepared by eight intravenous injections, each containing 3×10^6 rabbit thymocytes. The antiserum was inactivated and repeatedly absorbed with rabbit erythrocytes, liver, kidney, and testicular cells until the serum no longer killed normal rabbit testicular cells. This absorbed antiserum reacted with all leucocytes. Goat anti rabbit testis serum (ATeS) was prepared by intradermal injections with homogenised rabbit testes, 10 injections in incomplete and four in complete Freund's adjuvant. The antiserum was inactivated and repeatedly absorbed with rabbit spleen cells until it no longer killed lymphoid cells. After absorption the antiserum was cytotoxic only for testicular cells and spermatozoa.

The cytotoxicity test was performed as follows: to each 2×10^6 cells in 1 ml of medium was added 0·02 ml of undiluted antiserum. The mixture was incubated for 30 minutes at room temperature, and 0·1 ml of rabbit complement (diluted 1/2) was added. This mixture was incubated for an additional 60 minutes at 37°C and then diluted with 4 ml of medium. One drop of the cell suspension was mixed with one drop of 0.4% trypan blue to determine the cytotoxic effect of the antiserum.

The cell suspension was then layered on a standard mixture of Ficoll-Hypaque[7] and centrifuged at 400 × *g* for 30 minutes. The viable cells recovered at the interphase of the gradient were washed twice and resuspended in RPMI 1640 medium containing antibiotics and 10% inactivated fetal calf serum.

In vitro mitogenic stimulation

Triplicate samples of testicular cell suspension (TCS), TCS depleted of testicular cells (TCd-TCS) or TCS depleted of leucocytes (LCd-TCS), each containing 10^6 cells per ml of medium, were distributed in 0·2 ml portions into microplates. Either purified concanavalin A grade IV (Sigma Chemical Company, St Louis, Missouri, United States) 4 μg per well or undiluted anti allotype (anti Ab4 or anti Ab9) serum 10 μl per well was added. The antisera against light chain allotypic determinants (Ab4 and Ab9) were prepared as described.[8] The optimal conditions for stimulation by all mitogens were determined in preliminary experiments. Control unstimulated cultures received only PBS.

The cultures were incubated for 48 hours in 5% carbon dioxide. Six hours before harvesting the samples, 0·5 μCi of tritiated thymidine (New England Nuclear, Boston, Massachusetts, United States) was added per well and the cells were harvested with a multiple cell harvester. The tritiated thymidine uptake was determined in a liquid scintillation spectrophotometer (Mark III, Tracor Analytic, Elk Grove Village, Illinois, United States). The counts per minute (cpm) of triplicate samples were averaged, and the net cpm was determined by subtracting the mean cpm of the non-stimulated cultures from that of the stimulated cultures. The stimulation index (SI) was determined as the quotient of the mean cpm of the stimulated cultures divided by that of the non-stimulated cultures.

Result

CONDITION OF TESTES AT TIME OF REMOVAL

No macroscopic changes were observed during the

first 48 hours after infection, excluding the possibility of a non-specific infection with other organisms. The rabbits infected with *T pallidum* showed a typical course of infection: on day 6 the testes from all animals were noticeably infiltrated; on day 11 a well developed orchitis was observed; and on day 24 gummatous tissue (1-1·5 cm) was palpated in two of three animals.

In animals infected with washed *T pallidum,* although the inoculum contained more organisms, the inflammatory process was delayed. The testes were only slightly infiltrated on day 11, comparably with those on day 6 in the group infected with *T pallidum,* and never reached the degree of inflammation observed in that group. On day 24, however, periorchitis was observed and small (about 0·5 to 0·8 cm in diameter) gummatous tissue could be palpated in both animals.

In the testes of rabbits injected with heat killed *T pallidum,* Reiter treponeme, or normal rabbit testis extract no macroscopic evidence of change was seen at any time.

NUMBERS OF TREPONEMES

The numbers of treponemes in the fresh testicular imprints of animals infected with *T pallidum* or washed *T pallidum* increased progressively from days 3 to 11 and decreased thereafter. Table I shows, however, that treponemes were definitely present and still in considerable numbers on day 24. The large inoculum of washed *T pallidum* may account for the larger number of organisms seen in that group on days 3 and 6. The non-pathogenic organisms, heat killed *T pallidum* and Reiter treponeme, must have been cleared by the host rather quickly since none was seen, even on day 3.

TABLE I *Numbers of treponemes in testes infected with T pallidum or washed T pallidum*

Days after infection	*Suspension injected*	*Treponemes (× 10⁶/ml)*	
		Range	*Average*
3	*T pallidum*	0·6-2·4	1·4
	washed *T pallidum*	6·3-8·4	7·3
6	*T pallidum*	8·4-18·0	8·7
	washed *T pallidum*	40-210	82
11	*T pallidum*	Innumerable	
	washed *T pallidum*	Innumerable	
24	*T pallidum*	2·1-21·0	10
	washed *T pallidum*	0·9-4·2	1·1

HISTOLOGICAL EXAMINATION

Figure 1 shows sections of the testes of control animals injected with normal rabbit testis extract and of rabbits infected with *T pallidum.* The former appeared normal with uniform seminiferous tubules with a thin basement membrane lined with a single cell layer of spermatogonia (dark dots) and one or two layers of primary and secondary spermatocytes and spermatids. There were few venules, arterioles, or interstitial Leydig's cells between the tubules.

On day 3 animals infected with *T pallidum* showed histological features similar to those of controls, except that the infected group had microscopic areas of acute orchitis, characterised by an infiltration of polymorphonuclear leucocytes (75%), histiocytes (15%), and lymphocytes (10%), with occasional plasma cells especially in the perivascular areas. On day 6 the infected animals showed moderate inflammatory changes in the interstitial and vascular areas. The polymorphonuclear cells had decreased to about 5%, while the proportions of lymphocytes and plasma cells (black arrows) increased to 75% and 10% respectively. Histiocytes constituted about 10% of the cells. On day 11 the infected animals showed pronounced mononuclear cell infiltration in the interstitial and vascular areas, with plasma cells and lymphocytes in similar numbers (each about 45%) and some histiocytes (10%). Conspicuous atrophy of the seminiferous tubules and suppressed spermatogenesis were evident. On day 24 the testes of infected animals showed infiltration of lymphocytes (80%), plasma cells (10%), histiocytes (8%), and polymorphonuclear cells (2%). Scattered lymphoid follicular hyperplasia was evident throughout the interstitium. The lymphoid follicles varied in size, with typical lymphoblastic hyperplasia and prominent germinal centres surrounded by paracortical histiocytes. There was considerable interstitial fibrosis with pronounced atrophy of the seminiferous tubules.

Figure 2 shows that the testes of rabbits infected with washed *T pallidum* were normal on day 3. On days 6 and 11, however, they were infiltrated with cells similiar to those seen in the *T pallidum* infected group, although the infiltration was less pronounced and fewer plasma cells were seen. The interstitial tissue was uniformly infiltrated with inflammatory cells but contained no connective tissue, ground substance, or fibroblastic activity. On day 24 a thick tunic was observed which was infiltrated with lymphocytes, plasma cells, and macrophages showing pronounced fibrosis. Seminiferous tubules were extremely atrophic. Interstitial tissue was infiltrated with lymphocytes and fibroblasts. Focal areas of lymphoblastic proliferation, resembling lymph nodes, were also seen.

Testes of animals injected with heat killed *T pallidum* and *T phagedenis* Reiter did not show noticeable abnormalities.

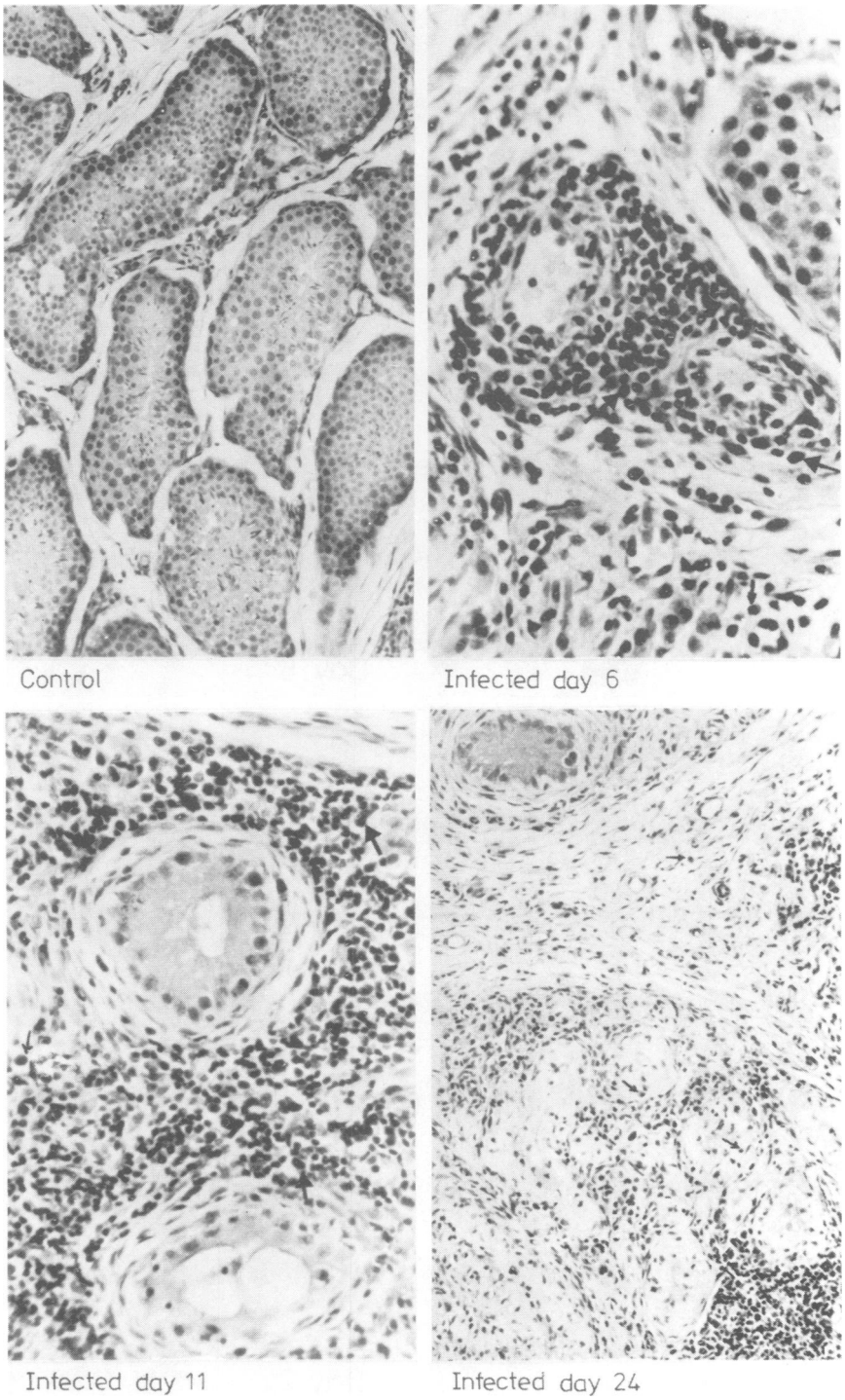

FIG 1 *Histological examination (haematoxylin and eosin staining) of testes from control rabbits (50 × magnification) and from rabbits infected with T pallidum removed on Days 6, (125 ×), 11 (130 ×), and 24 (50 ×) after infection.*

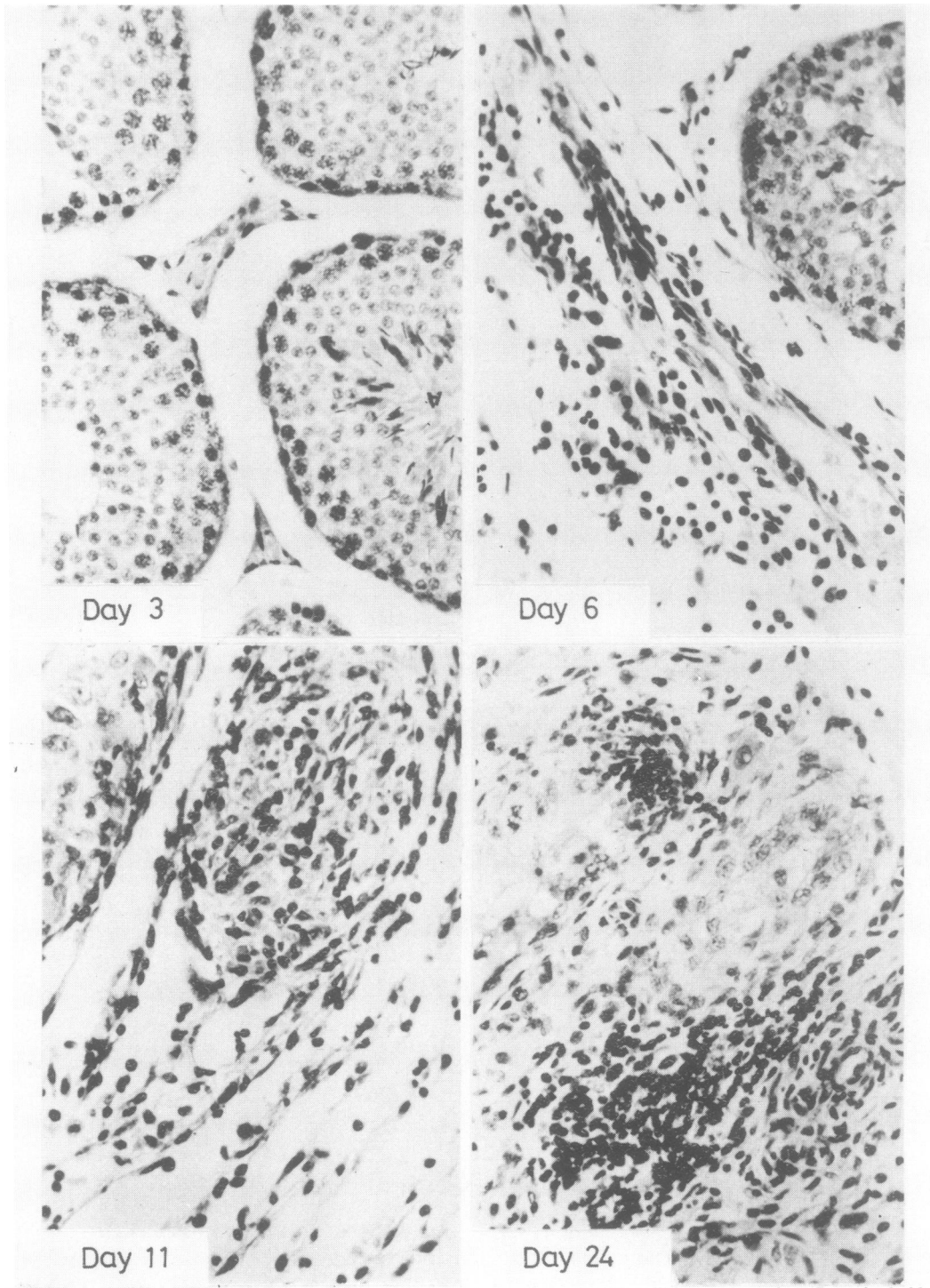

FIG 2 *Histological examination of testes from rabbits infected with washed T pallidum removed on Days 3, 6, 11, and 24 (all 320 × magnification) after infection.*

TREPONEMAL ANTIBODY

Until day 11 after infection with *T pallidum* no treponemal antibodies were detected in the serum. Table II shows that by day 6, however, the testicular fluid from three out of four rabbits had a titre of <1/5. On day 11 the titre was three times higher in the testicular fluid than in the serum. On day 24 the titre in the testicular fluid remained the same while that in the serum had increased. The animals infected with washed *T pallidum* responded from day 11 with treponemal antibody production; the titres were higher in the serum than in the testicular fluid. The rabbits infected with heat killed *T pallidum* responded on day 24 (not shown in the table) but only in the testicular fluid (titre 1/15). No treponemal antibodies were found in rabbits injected with Reiter treponeme or normal rabbit testis extract.

TABLE II *Results of fluorescent treponemal antibody (FTA) tests with serum and testicular fluid of rabbits infected with T pallidum*

Days after infection	*Suspension injected*	*Average titre in:* *Serum*	*Testicular fluid*
3	*T pallidum*	<1:5	<1:5
	washed *T pallidum*	<1:5	<1:5
6	*T pallidum*	<1:5	1:5
	washed *T pallidum*	<1:5	<1:5
11	*T pallidum*	1:45	1:135
	washed *T pallidum*	1:45	1:15
24	*T pallidum*	1:405	1:135
	washed *T pallidum*	1:1215	1:135

IDENTIFICATION OF T AND B CELLS BY IMMUNOFLUORESCENCE

Table III shows that in the infected testes there was a wide variation in the number of cells stained by each antiserum. Slides of three out of eight rabbit testes contained more immunoglobulin positive cells (B cells) than T cells. Two slides per rabbit and 500-700 microscope fields per slide were examined. The results are expressed as an average per 100 fields.

TABLE III *Numbers of T and B lymphocytes in the testes of rabbits infected with T pallidum*

Inoculum	*Day orchitis observed*	*No of rabbits*	*No of Lymphocytes (range of means/100 fields)* *T*	*B*
T pallidum 10×10^6/testis	11	4	2-15	7-29
T pallidum 48×10^6/testis	9	4	5-30	5-64
Normal testicular fluid		2	6-8	2-3

MITOGENIC ACTIVITY

Table IV shows the mitogenic responses of testicular cell suspension (TCS) and TCS depleted of testicular cells (TCd-TCS) or leucocytes (LCd-TCS) to concanavalin A or allotype antibodies (Ab4 or Ab9). The responses were determined for pairs of testes obtained from days 7 to 11. A variable but significant response to the T cell mitogen concanavalin A and the B cell mitogen anti Ab4 was obtained with cells of most of the infected testes. Maximal proliferative responses were found to concanavalin A on day 11 but the response to anti Ab4 varied. Treatment of testicular cell suspension with goat anti rabbit testes serum did not always enhance the proliferative response to the mitogens. Treatment with goat anti rabbit thymocyte serum, however, always inhibited the cellular response to both mitogens. The cellular response to anti Ab9, an antigen not present in the rabbits' B cells, was negative in all animals. The responses to both mitogens of testicular cell suspension and TCS depleted of testicular cells or leucocytes were less than those of peripheral blood lymphocytes from five normal and six infected animals. Testicular cell suspension from normal rabbit testes did not respond to T or B cell mitogens.

Discussion

Injection of rabbit testes with virulent *T pallidum* caused a sequence of histological changes similar to those reported by other investigators.[9-12] In this study plasma cells were definitively identified as one predominant component of the lymphocytic infiltration throughout the experimental period. This was determined by histological examination, localised production of antibodies, immunofluorescent staining, and mitogenic stimulation of the cells extracted from the infected testes. While B cell response to specific mitogen anti Ab4 was roughly constant in all testicular cell suspension preparations from day 7 on, the T cell response to concanavalin A increased steadily to a maximum on day 11 as did the number of *T pallidum* organisms, which were still present in significant numbers on day 24 after infection.

The way the lymphocytic infiltration influences the local growth of *T pallidum* and the course of syphilitic infection is not clear although it has been a matter of speculation by several investigators. Lukehart *et al*[2] observed a mononuclear cell infiltration consisting almost entirely of T lymphocytes and macrophages with few or no plasma cells. Although the inoculum used by these authors was four to five times larger than the one used in our study, by day 17 these workers found no treponemes, either by indirect immunofluorescence with human anti-

TABLE IV *Responses of peripheral blood lymphocytes, testicular cells from control rabbits, and from those infected with T pallidum to T and B cell mitogens*

Days after infection	Cells*	Concanavalin A		Antibodies to Ab4 allotype		Antibodies to Ab9 allotype	
		Net cpm	SI	Net cpm	SI	Net cpm	SI
7	TCS	2 538	1·9	1 460	1·5	−756	0·7
	TCd-TCS	2 993	1·9	4 340	2·3	−546	0·8
	LCd-TCS	2 702	0·5	1 126	1·2	−407	0·3
7	TCS	1 012	2·0	4 234	5·2	−189	0·8
	TCd-TCS	825	1·9	4 917	5·7	−121	0·9
	LCd-TCS	132	1·3	−176	0·6	−320	0·3
9	TCS	2 416	2·7	1 369	1·5	Nil	
	TCd-TCS	3 877	11·0	1 597	2·0	Nil	
	LCd-TCS	287	1·8	−31	0·9	Nil	
9	TCS	24 013	6·0	5 833	2·2	Nil	
	TCd-TCS	41 601	13·3	7 629	3·3	Nil	
	LCd-TCS	1 875	2·8	177	1·2	Nil	
10	TCS	7 295	9·8	338	1·4	Nil	
	TCd-TCS	7 899	6·1	914	1·6	Nil	
	LCd-TCS	780	1·0	−200	0·8	Nil	
10	TCS	8 457	5·5	250	1·1	Nil	
	TCd-TCS	9 500	6·1	1 179	1·8	Nil	
	LCd-TCS	700	0·7	128	0·3	Nil	
11	TCS	60 267	36·0	3 878	3·2	25	0·9
	TCd-TCS	78 319	73·0	4 387	5·0	−95	0·9
	LCd-TCS	972	1·7	336	0·8	−280	0·7
11	TCS	77 996	53·0	5 546	4·4	−3	1·0
	TCd-TCS	69 421	27·2	11 043	5·3	−200	0·8
	LCd-TCS	450	1·4	210	0·8	−350	0·7
Normal	TCS	135	1·0	−20	1·0	−101	0·7
Normal	TCS	40	1·0	10	1·0	75	1·0
9 to 11 (n=6)	PBL	191 003	112·0	50 802	35·8	−2 050	0·7
Normal (n=5)	PBL	153 877	77·2	88 383	32·1	−1 087	0·9

*TCS = testicular cell suspension; TCd-TCS = testicular cell-depleted TCS; LCd-TCS = leucocyte-depleted TCS; PBL = peripheral blood lymphocytes; SI = stimulation index.

treponemal antiserum as first reagent or by direct immunofluorescence with labelled anti rabbit IgG antiserum. From this and their previous study of the proliferative response of lymph node and spleen cells from *T pallidum* infected animals,[13] the authors concluded that in primary syphilis T lymphocytes were functional in their response to *T pallidum*, and that T cells together with macrophages were responsible for the early clearance of treponemes from the site of infection. Our results disagree with those of Lukehart *et al* as plasma cells were consistently found in our experiments and have been observed by other investigators in practically all stages of natural infection with *T pallidum*.[14] We cannot exclude the possibility that some part is played by the plasma cells and their products at the site of infection, although the mechanism is unknown at present.

The speculation of Lukehart *et al* on the specificity of the cellular response and the immunological competence of the T cells at the site of infection[2 13] does not seem appropriate as antigens of non-pathogenic treponemes were not included as controls in any of their studies, and no direct evidence was provided of the immunological competence of the mononuclear cells. As we report[4] the immunological competence of the mononuclear cells, particularly T cells, infiltrating the infected testis is impaired. Finally, the failure of Lukehart *et al* to demonstrate treponemes 17 days after infection is probably due to their use of tissue sections processed as for pathological examination. Fresh imprints used in our experiments showed clearly that treponemes were not so rapidly and totally eliminated from the testes despite the early local humoral response and the increasing lymphocytic infiltration.

The inclusion of animals infected with washed and heat killed *T pallidum* as controls provided additional information on the kinetics and determining factors of cellular infiltration at the site of inoculation. It has been suggested that the large amount of mucoid material produced during inflammation in animals infected with *T pallidum* may coat and protect them from the host's defence mechanism.[15-17] Repeated washing of treponemes removes most of the protective layer and injection of such treponemes should make them an easy target for the host's defence mechanism.[18] In our experiments using

washed *T pallidum* this seemed not to be the case. An inoculum of washed *T pallidum* six times larger than that of untreated *T pallidum* elicited a relatively mild, much delayed, and less intense testicular infiltration containing few plasma cells, and with a lower titre of antitreponemal antibodies than in the serum. Although part of the inoculum escaped rapidly from the testes into the systemic circulation, many treponemes (in proportion to the larger inoculum) were seen in smears on day 3. The consistency of these results in all animals infected with washed *T pallidum* strongly suggests that the early cellular and humoral response in the group infected with untreated *T pallidum* may be triggered not primarily by the *T pallidum* but by the inflammatory substances of the testicular fluid injected with the treponemes. Those substances (for example, lymphokines or immune complexes) may directly or indirectly induce chemotactic activity facilitating the early cellular infiltration of the testis. With local multiplication of the treponemes and de novo synthesis of antibodies by the attracted plasma cells, more complexes are formed, complement is activated, and more chemotactic factors are released, attracting new cells to the site of infection.

If this assumption is correct, the inflammatory process in the testes may be considered the result of a joint action initiated by the testicular inflammatory substances and enhanced by those released during local treponemal growth and the developing immune response. The absence of testicular inflammatory substances in washed *T pallidum* suspension would explain the delay and low degree of testicular inflammation in the animals infected with that suspension. Delayed and slight inflammation is consistent with the poor immunogenicity of *T pallidum* and with the lack of chemotactic activity in washed *T pallidum* preparations.[19] In animals infected with washed *T pallidum* as opposed to those infected with untreated *T pallidum,* treponemal antibodies appear simultaneously in serum and in testicular fluid. This finding correlates with the delayed arrival and low number of locally attracted plasma cells. Thus while local growth of treponemes seems directly related to and affected by the cellular infiltration no conclusion as to the type of cell(s) involved can be drawn from the present experiments. Both T and B cells might function equally in this process.

The control animals injected with heat killed *T pallidum* furnished additional information. A local humoral response was observed on day 24 but only in testicular fluid. Treponemes were never detected. Despite the apparent absence of cellular infiltration, we assume that the local production of antibodies could originate only from plasma cells attracted to the site of inoculation. Since washed *T pallidum* proved to be a poor chemoattractant, heat stable chemotactic substances present in the inoculum may explain the cellular infiltration responsible for the local immunological response. The absence of treponemal antibodies in testicular fluid 11 days after infection excluded any possibility of passive transfer with the inoculum.

In summary, the contrasting histopathological results for the groups infected with *T pallidum* and washed *T pallidum* as opposed to heat killed *T pallidum* and Reiter treponemes clearly indicated the difference in immunological response to pathogenic as opposed to non-pathogenic treponemes. The latter are quickly and effectively removed from the site of inoculation whereas pathogenic treponemes stay and multiply for a relatively prolonged time. This increase occurs despite a local cellular and humoral response, the nature of which may be largely dependent on biologically active substances accompanying the infectious inoculum.

References

1. Yeagle N, Kalinka C, Nakeeb S, Wicher V, Wicher K. Immunopathology of rabbit testes infected with *Treponema pallidum*. *Abstracts, Annual Meeting, American Society of Microbiology* 1978; **B20**, p17.
2. Lukehart SA, Baker-Zander SA, Lloyd RMC, Sell S. Characterization of lymphocyte responsiveness in early experimental syphilis. II Nature of cellular infiltration and *Treponema pallidum* distribution in testicular lesions. *J Immunol* 1980; **124**: 461-7.
3. Wicher V, Wicher K. Studies of rabbit testes infected with *Treponema pallidum*. II Local synthesis of antibodies. *Br J Vener Dis* 1983; **59**: 359-63.
4. Wicher V, Wicher K. Studies of rabbit testes infected with *Treponoma pallidum*. III Immunosuppressive activity of the infiltrating mononuclear cells. *Br J Vener Dis* 1984; **60** (in press).
5. Magnuson HJ, Eagle H, Fleischman R. The minimal infectious inoculum of *Spirochaeta pallida* (Nichols strain), and a consideration of its rate of multiplication in vivo. *American Journal of Syphilis, Gonorrhea and Venereal Diseases* 1948; **32**: 1-18.
6. Hanson AW, Cannefax GR. Recovery of *Treponema* and *Borrelia* after lyophilization. *J Bacteriol* 1964; **88**: 811.
7. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* [Suppl 97] 1968; **21**: 77-89.
8. Dubiski S. Immunochemistry and genetics of a "new" allotype specificity Ae14 of rabbit G immunoglobulins: recombination in somatic cells. *J Immunol* 1969; **103**: 120-8.
9. Azar HA, Pham TD, Kurban AK. An electron microscopic study of a syphilitic chancre. *Archives of Pathology* 1970; **90**: 143-50.
10. Gregoriew PS, Saratow KGJ. The histologic structure of syphilitic lesions of rabbits. *American Journal of Syphilis, Gonorrhoea and Venereal Diseases* 1928; **12**: 67-81.
11. Hardy PH Jr, Graham DJ, Nell EE, Dannenberg AM Jr. Macrophages in immunity to syphilis: suppressive effect of concurrent infection with *Mycobacterium bovis* BCG on the development of syphilitic lesions and growth of *Treponema pallidum* in tuberculin-positive rabbits. *Infect Immun* 1979; **26**: 751-63.
12. Scott V, Dammin GJ. Morphologic and histochemical sequences in syphilitic and tuberculous orchitis in the rabbit. *American Journal of Syphilis, Gonorrhea and Venereal Diseases* 1954; **38**: 189-202.

13. Lukehart SA, Baker-Zander SA, Sell S. Characterization of lymphocyte responsiveness in early experimental syphilis. I In vitro response to mitogens and *Treponema pallidum* antigens. *J Immunol* 1980;**124**:454-60.
14. Pund ER, von Haam E. Spirochetal and venereal disease. In: Pathology, Anderson WAD, ed. *Pathology*. 3rd ed. St Louis: CV Mosby, 1957;264-291.
15. Christiansen S. Protective layer covering pathogenic treponemes. *Lancet* 1963;i:423-5.
16. Penn WC. Avoidance of host defences by *Treponema pallidum* in situ and on extraction from infected rabbit testes. *J Gen Microbiol* 1981;**126**:69-75.
17. Turner TB, Hollander DH. Biology of the treponematoses. *Series No 35*. Geneva: WHO, 1975;35-87.
18. Zeigler JA, Jones AM, Jones RH, Kubica KM. Demonstration of extra-cellular material at the surface of pathogenic *T. pallidum* cells. *Br J Vener Dis* 1976;**52**:1-8.
19. Kalinka C, Belani R, Wicher V, Wicher K. Chemotaxis of peritoneal neutrophils of *Treponema pallidum*-infected rabbits. *Abstracts, Annual Meeting, American Society of Microbiology* 1980;**B41**: p 24.